Suppression of RNA Interference by Virus-Associated RNA of Adenovirus Serotypes Ad4, Ad5, Ad11 and Ad12

Xiao Wang

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Biology Education Centre and Department of Medical Biochemistry and Microbiology, Uppsala University
Supervisor: Göran Akusjärvi
Abstract

RNA Interference (RNAi) is the process of gene silencing that is induced by double-stranded RNA, in a homology-dependent manner. Human adenovirus 5 (Ad5) produces highly-structured virus-associated (VA) RNAs that resemble precursors to microRNA, which are cleaved by Dicer into virus-associated RNA-derived small RNAs (mivaRNAs) during a lytic infection. These mivaRNAs are then functionally incorporated into the RNA-induced silencing complex (RISC), thereby inhibiting RNAi. Related studies to date have only focused on human Ad5. There are 56 serotypes of human adenoviruses. To investigate whether they share the same RNAi suppression mechanism with Ad5, we selected adenovirus type 4, 11 and 12 (belonging to subgroup E, B and A respectively) to infect 293-Ago2 cells. mivaRNAs in immunopurified RISC from cytoplasmic extracts at 24 hours post-infection were analyzed by Northern blotting. The results showed that also Ad4, Ad11 and Ad12 produce VA RNA-derived mivaRNAs, which are efficiently incorporated into RISC complexes. Thus, suppression of the RNAi pathway by mivaRNAs is probably a shared gene regulatory mechanism among different adenovirus serotypes. Here we also quantified Ad5 VA RNAI in human 293 cells and verified that during late infection, the synthesis the VA RNAI rapidly accumulate to very high level, around 2.6-4.0× 10^6 Ad5 VA RNAI molecules per cell.
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1 Introduction

1.1 RNA Interference

When double-stranded RNA (dsRNA) is introduced into a living cell, it will induce target gene silencing in a homology-dependent manner through a process called RNA interference (RNAi), which is a universal and diverse regulatory system shared by virtually all eukaryotic organisms. Napoli et al. first observed the phenomenon of RNA silencing in 1990 (Napoli, et al., 1990), whereas Craig C. Mello and Andrew Fire coined the term RNAi and revealed the mechanism in Caenorhabditis elegans (Fire, et al., 1998), which earned them a Nobel Prize in Physiology or Medicine in 2006. RNAi is a significant gene regulation machinery and plays important roles both in physiology and pathology. It has been widely used as an experimental tool to create model systems, recognize molecular targets and potentially mediate new therapeutic treatments.

The trigger dsRNA can be both exogenously derived (such as virus-encoded RNAs from infection or dsRNAs delivery by biotechniques) and endogenously expressed (Hutvagner, 2005). In an RNAi pathway, the initiating dsRNA will be cleaved into 20-25 nucleotides (nt) small RNAs by an RNase III type enzyme named Dicer. One strand of the small RNA is then integrated into an endoribonuclease-containing effector complex called the RNA-induced silencing complex (RISC). The single-strand small RNA acts as a guide strand and base-pairs to its target RNA, resulting in mRNA degradation or translational repression (Kim, 2005).

The small RNAs that endow the RNAi machinery with target specificity can be classified into two types based on their origin: small interfering RNAs (siRNAs) and microRNAs (miRNAs). siRNAs primarily originate from perfectly base-paired long dsRNAs, though several endogenous sources of siRNAs (such as repeat-associated siRNAs derived from repetitive genome sequences) have now been identified. miRNAs are generated from the dsRNA part of stem-loop structured endogenous transcripts called pri-mRNAs. The primary transcripts are processed by the enzyme Drosha in the nucleus and then exported to cytoplasm for Dicer processing. miRNAs and siRNAs henceforth share similar pathways to mediate RNA silencing (Fig. 1).

The ultimate fate of a target RNA is determined by the sequence complementarity of the small RNA and its target RNA. Typically, siRNAs are perfectly base-paired to its specific target and lead to mRNA cleavage and degradation whereas miRNAs are partially complementary to its targets and induce translational inhibition (Fig. 1). miRNAs were discovered through their critical fine-tuning regulated functions in cell differentiation, development, cell proliferation as well as oncogenesis. It is estimated that over 1000 miRNAs encoded by human genome may participate in the regulation of 60% of mammalian genes (Bentwich, et al., 2005; Burge, et al., 2009).
1.2 Key proteins in RNAi

1.2.1 Dicer enzyme

The RNase III family enzyme Dicer universally exists in eukaryotic organisms, which enable to cleave dsRNA or pre-miRNA into small RNAs with 2-nt 3’ overhangs. Its structure contains a PAZ domain that recognizes the 3’ overhang produced by Drosha and a dsRBD domain, which binds to long dsRNA (Zhang, et al., 2004). Besides, RIIIDa and RIIIDb are its catalytic domains. When capturing a substrate, Dicer will dimerize and form a processing center, generating siRNA or miRNA duplexes with 3’-hydroxyl and 5’-phosphate overhangs (Bernstein, et al., 2001).

1.2.2 Argonaute proteins

Argonaute proteins are catalytic components in RISC complexes or homologous complexes, which act as effectors in the RNAi pathway. Single strands of small RNAs (siRNAs or miRNAs) bind to Argonaute proteins and guide the proteins to cleave the target mRNAs which are complementary to the small RNA fragments (Rand, et al., 2005). Argonaute proteins are divided into two sub-families: Ago and Piwi.
Eight Argonaute proteins have been identified in human: hAgo1/eIf2C1, hAgo2/eIf2C2, hAgo3/eIf2C3, hAgo4/eIf2C4, PIWIL1, PIWIL2, PIWIL3, PIWIL4. PAZ and PIWI are two conserved domains in Argonaute structure. The PAZ domain is responsible for recognizing the 3’ overhangs of small RNAs processed by Dicer (Lingel, et al.,2004). PIWI endows Argonaute protein RNaseH characteristics (Parker, et al.,2004).

Since it has been shown that human Argonaute2 mediates cleavage of mRNA targeted by miRNAs and siRNAs, a 293-Ago2 cell line which stably expressing a FLAG/HA epitope tagged Ago2 protein was established to aid in the investigation of the interaction between small RNAs and RISC (Xu, et al.,2007).

1.3 Adenovirus

Adenovirus was first isolated from human adenoidal tissues in 1953, hence the name (Rowe, et al.,1953). Adenoviruses commonly cause diverse diseases including respiratory infections, eye infections and gastroenteritis. Apart from their interests as pathogens, adenoviruses are important model systems for understanding the cellular and molecular mechanisms including pre-mRNA splicing (1993 Nobel Prize) (Berget, et al.,1977), cell cycle control and small RNA regulation as is the interest in this thesis. Recently, the use of adenoviruses as vectors in gene therapy (especially cancer therapy) and vaccination has increased dramatically (Imperiale, et al.,2004).

1.3.1 Structure and taxonomy

Adenoviruses are non-enveloped, medium-sized (70-90 nm) icosahedral viruses with double-stranded DNA genome containing between 22-40 genes. The genome of adenovirus is well characterized and consists of five early transcription units (E1A, E1B, E2, E3, and E4), two delayed early units (pIX and Iva2) and one major late transcriptional unit (L1-L5) (Fig. 2). Among the 40-50 proteins encoded by adenovirus, around 12 are structural proteins of the virion, which contains 240 hexons, 12 pentons and 1 fiber on each penton base. The fiber is used for attachment of the virus to its host cell (Rux, et al.,2004).

Human adenoviruses constitute a large group in the Adenoviridae family. So far, there are 56 accepted human adenovirus serotypes described (genus Mastadenovirus) that are classified into seven subgroups (A-G) based on their surface antigens, oncogenicity and genomic sequences (Adhikary, et al.,2001;Jones, et al.,2007) (Table 1). Subgroup A, F and G species primarily infect the gastroenteric tract. Ad11, Ad34 and Ad35 of subgroup B are associated with urinary tract and kidney infection. Subgroup C commonly causes endemic infection in children whereas serotypes Ad8, Ad19 and Ad37 in subgroup D cause epidemic keratoconjunctivitis (Dart, et al.,2009). Acute respiratory disease (ARD) is most often associated with Ad4 and Ad7.

Most work to date has been done on serotype Ad2 and the closely related Ad5 within subgroup C. Ad12 has been widely used in cancer research due to its high oncogenicity in rodents (Trentin, et al.,1962). Ad11 has been developed to a new
vector candidate for gene therapy due to its lower serum prevalence and better target capability (Mei, et al., 2003).

Fig. 2. Transcription of the adenovirus genome. The genome consists of early transcription units (E1A, E2B, E2, E3 and E4), delayed early units (pIX and IVa2) and late transcriptional unit (L1-L5). VA genes are located close to unit 30. Image provided by Prof. G. Akusjarvi.

Table 1. Human adenovirus species classification

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Serotype</th>
<th>Sites of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12, 18, 31, 14</td>
<td>Intestine</td>
</tr>
<tr>
<td>B</td>
<td>3, 7, 11, 14, 16, 21, 34, 35, 50, 55</td>
<td>Urinary tract, upper respiratory tract, kidney</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
<td>Respiratory tract</td>
</tr>
<tr>
<td>D</td>
<td>8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51, 53, 54, 56</td>
<td>Eye, intestine</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>Respiratory tract</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>Intestine</td>
</tr>
<tr>
<td>G</td>
<td>52</td>
<td>Intestine</td>
</tr>
</tbody>
</table>
1.3.2 The adenovirus VA RNAs

The virus-associated (VA) RNA is a class of low-molecular weight (around 150-180 nt) (Ma, et al., 1996), non-coding RNA found in adenovirus-infected cells. A majority of human adenoviral serotypes encode two VA RNA species called VA RNAI and VA RNAII while the other serotypes generate only a single VA RNA species, which structurally and functionally appears to resemble VA RNAI (Ma, et al., 1996). Both VA RNA genes are located close to map unit 30 (upstream of L1 co-termination family) in the genome and transcribed by RNA polymerase III from tRNA-like promoters in rightward direction (Akusjarvi, et al., 1980) (Fig. 2).

The human VA RNA gene sequences among different serotypes display considerable variability. Only around one-third of the nucleotides are conserved across all serotypes. Nonetheless, all of the VA RNAs adopt stable hairpin-shaped secondary structure consisting of an apical stem-loop, a central domain, and a terminal stem, which are important for their functions. (Ma, et al., 1996) The VA RNAs are heterogeneous at both their 3’ and 5’ strands due to variations in initiation and termination sites of transcription (Harris, et al., 1978).

The VA RNAs start to be synthesized at the early phase of infection and accumulate to extreme high levels during late infection. The synthesis of Ad2 VA RNAI can be up to $10^8$ molecules per cell, roughly the abundance of ribosomes. VA RNAII is expressed at 20-fold lower levels (around $5 \times 10^6$), which still is a very high level compared to other RNAs in a cell (Soderlund, et al., 1976). It is suggested that VA RNAI is essential for efficient viral mRNA translation by blocking the interferon-induced antiviral response. The central domain of VA RNAI binds to the dsRNA-dependent protein kinase (PKR) and inhibits it from phosphorylating eukaryotic translation-initiation factor 2α (eIF2α), thereby rescuing the translational capacity in infected cells (Sarnow, et al., 2006) (Fig. 3). The ability of VA RNAs to inhibit PKR varies among different serotypes and VA RNAII has no function as a PKR inhibitor (Ma, et al., 1996).

In this study we have focused on another significant function of the VA RNAs, which was revealed in recent years. Thus, during a lytic infection the VA RNAs are processed into small RNAs that are incorporated into RISC and have a subverting effect on the RNAi machinery (described below).

1.3 Virus-host interaction

1.3.1 Antiviral silencing

All viruses, including those with DNA genomes, produce dsRNAs as replication intermediates or side products of transcription during their replication cycle. When the trigger dsRNAs are larger than 50 bp, they will active the interferon-PKR response in mammals (Sarnow, et al., 2006). Since plants have no innate immune system, a virus-induced gene silencing (VIGS) system is adopted as the natural response to dsRNA. Those viral dsRNAs are processed into small RNAs and loaded
into RISC complexes, leading to specific recognition and silencing of viral RNAs (Ruiz, et al., 1998). This RNAi anti-viral mechanism is broadly found in plants and insects, however, the first indication that RNA silencing might also serve as antiviral response in animals was proposed in 2002 after the discovery of the B2 protein in flock house virus (FHV), which functions as a RNA silencing suppressor (Ding, et al., 2004). Since then, an increasing number of suppressors of RNA silencing (VSR) have been found in mammalian cells.

Interestingly, different viruses exploit a diverse array of mechanisms to fight the anti-viral silencing system. The NS1 protein of influenza virus binds to dsRNAs and siRNAs, thereby avoiding them from processing and incorporating into RISC (Li, et al., 2004). HcPro of potyvirus recruits the rgsCaM protein, which is an endogenous silencing suppressor (Vance, et al., 2000). Other viruses can, in fact, exploit the RNAi machine for their own benefit. For example, Simian virus 40 (SV40) encodes small RNAs which can induce gene silencing of early transcripts for T antigens, leading to a reduction of T antigen synthesis, therefore protecting SV40 from cytotoxic T lymphocytes (Sullivan, et al., 2005). Besides, Hepatitis C virus (HCV) utilizes host liver specific microRNA (miR-122) to enhance virus replication (Jopling, et al., 2005).

1.3.2 Adenoviral suppression of RNAi

Studies on the mechanisms of adenoviral suppression of the RNAi pathway have so far focused on serotype 5 (Ad5). The adenovirus VA RNAs are highly structured and adopt secondary structure that resembles hairpin-shaped miRNA precursor RNAs (pre-miRNAs). During an Ad5 infection, both VAI and VAII are processed into miRNA-like small RNAs (so called mivaRNAs) by the Dicer enzyme. Subsequently one strand of the mivaRNA duplex is incorporated into RISC. Since the mivaRNAs are produced in large amounts in a virus infection, RISC complexes are saturated with the virus-derived small RNAs. As a consequence, the mivaRNAs act as competitive substrates that block RNA interference by suppressing the two enzymatic activities, Dicer and RISC (Fig. 3). The RISC-associated mivaRNAs behave as either functional siRNA or miRNA, depending on whether their sequences are fully complementary to their target RNAs (Andersson, et al., 2005). Sequestering the mivaRNAs with 2'-O-methyl-antisense oligonucleotides reduces virus viability, suggesting that the mivaRNAs possibly participate in gene regulatory events that are essential for viral replication and survival (Sarnow, et al., 2006).

Although the level of Ad5 VAI RNAI is 20-fold higher than VA RNAII in adenovirus-infected cells, VA RNAII has been shown to be preferentially assembled into RISC complexes (Xu, et al., 2007). The mivaRNAs derived from 3’ and 5’ terminal stems of the VA RNAs are asymmetrically incorporated into RISC. Also, the 3’ strand of VA RNAII account for majority of total small RNA pool in both the cytoplasm and RISC in adenovirus-infected cells (Xu, et al., 2009; Xu, et al., 2007).

Furthermore, VA RNAI can also competitively bind to the nuclear-export receptor exportin-5 on the nuclear membrane, thereby blocking the translocation of pre-miRNA from nuclear to cytoplasm (Fig. 3). As a result, VA RNAI has been proposed to inhibit miRNA accumulation in the cytoplasm (Lu, et al., 2004).
1.4 Aim

The inhibition of RNAi by VA RNAs is attractive but has only been studied in Ad5. Since more than 50 human adenovirus serotypes has been found in nature, we decided to test whether the production of mivaRNAs are shared between human adenoviruses belonging to different subgroups (Table 1). The primary aim of our project was to investigate whether the VA RNAs from adenovirus type 4, 11 and 12 can be processed by Dicer into mivaRNAs, and whether these mivaRNAs are efficiently incorporated into RISC.

Another task of the project was to quantify the level of VA RNAI expression in Ad5 under our experimental conditions. A previous study has suggested that Ad2 VA RNAI accumulates to very high levels ($10^8$ molecules per cell) during the late infection phase (Soderlund, et al.,1976). Our experiments aimed to reveal the specific Ad5 VA RNAI levels in human 293 cells.
2 Results

2.1 Quantification of the synthesis of Adenovirus 5 VA RNAI in human 293 cells

Adenovirus VA RNAI starts to be synthesized during the early phase of infection at a moderate level. As the viral DNA starts to replicate at the transition from the early to late phase, the number of DNA templates available for transcription increases and the copy number of VA RNA rapidly increases to very high levels (Soderlund, et al.,1976). To verify this statement and reveal the specific synthesis level of VA RNAI, we in vitro transcribed VA RNAI to produce a control RNA and quantified the VA RNAI synthesized by Ad5 in 293 cells. The Ad5 VA DNAI template for in vitro transcription was PCR amplified from plasmid pHindB. The control VA RNAI was produced by T7 transcription and resulted in the synthesis of $3.6 \times 10^{11}$ copies of VA RNAI per microliter. The RNA samples to be quantified were cytoplasmic RNA prepared from Ad5-infected 293 cells in late phase of infection. Quantification was based on signal intensity of Northern blotting analysis (Fig. 4).

![Image of Northern blot](image)

*Fig.4. Quantification of Ad5 VA RNAI in 293 cells.* A series of standard VA RNAI prepared through in vitro transcription from plasmid pHindB-Ad5VAI (Lane1-3) as well as cytoplasmic RNAs prepared 24h post-infection from Ad5-infected 293 cells (Lane 4,5) were separated on 8% denaturing acrylamide gel. $\gamma$-P-labeled DNA oligonucleotides complementary to 3' strand of mivaRNAI were used as probes.
The figure showed an approximately 160 nt Ad5 VA RNAI from both the in vitro synthesis (Lanes 1-3) and adenoviral expression in human 293 cells (Lanes 4 and 5). Based on the known copy number of the control VA RNA, we calculated that 4.2×10^{11} copies and 8.7×10^{11} copies of VA RNAI were obtained from 3.0 μg and 6.0 μg of total cytoplasmic RNA, respectively. Thus, there were approximately 1.4×10^{11} VA RNAI molecules per microgram of cytoplasmic RNA. Since 163.0 μg of cytoplasmic RNA was extracted from one 10-cm plate of 293 cells (9.0×10^{6} cells), we calculated that the total copy number of VA RNAI was 2.3×10^{13} copies/plate and 2.6×10^{6} copies/cell.

The experiment was repeated once with RNA samples extracted from cells infected by another batch of Ad5. The results were in good agreement and suggested a copy number of 4.0×10^{6} copies/cell. In conclusion, our data suggested that Ad5 VA RNAI in human 293 cells infected at a multiplicity of 50 FFU/cell accumulated to around 2.6-4.0×10^{6} copies/cell.

2.2 Ad4, Ad5, Ad11 and Ad12 all produce mivaRNAs but to various levels

Both Ad5 VAI and VAII have been well characterized as substrates for Dicer cleavage, generating mivaRNAs from either the 3’ or 5’ strand of the hairpin-structure (Xu, et al.,2007). To determine whether Ad4, Ad11 and Ad12 also produce mivaRNAs during infection, 293-Ago2 cells were infected with the four wild-type serotypes. Cytoplasmic RNAs were extracted at 24 hours post-infection and separated on an 8% denaturing polyacrylamide gel. More Ad12 extracts were loaded since we found that Ad12 expressed lower amounts of the VA RNAs compared to the other serotypes. Both VAI and VAII-derived mivaRNAs were detected by using oligo probes complementary to 3’ strand of mivaRNAI (Fig. 5) and 3’ strand of mivaRNAII (Fig. 6).

As shown in Fig. 5A, mivaRNAs (<34nt) were detected from all the four serotypes, indicating that Ad4, Ad11 and Ad12 VA RNAI (approximately 160 nt), like Ad5 VA RNAI, were cleaved by Dicer into mivaRNAs. However, the sizes of the mivaRNAs appeared to be slightly different, with the Ad5 mivaRNA being shorter than that of the other three serotypes. Furthermore, the cleavage efficiencies varied considerably among the four serotypes. The experiment was repeated using other batches of the viruses (displayed as infection A and infection B in Fig. 5B). According to the quantitative results, Ad12 and Ad11 VA RNA were more efficiently processed into mivaRNAs (14.2-20.3% and 16-20% respectively) compared to Ad5 and Ad4 VA RNAI (6-7.2% and 3.5-11.9% respectively). In the repeated experiment (Fig. 5B) the results were similar except that the Ad4 mivaRNA accumulated to a higher percentage in the second experiment.

Among the four selected serotypes, only Ad4 and Ad5 encode a VA RNAII gene. As shown in Fig. 6A, both Ad5 VARNNAI (161nt) and Ad4 VARNNAII (173nt) were cleaved, generating mivaRNAs with similar sizes. The experiment was repeated once (Fig. 6B). Quantification of the data indicated that Ad4 VARNNAII was cleaved with a higher efficiency (8-10.6%) compared to the Ad5 VA RNAII (4-5.32%) (Fig. 6B).
Fig. 5. Detection of mivaRNAs produced from VA RNAI of Ad4, Ad5, Ad11 and Ad12. (A) Cytoplasmic RNAs prepared 24h post-infection from Ad4, 5, 11, 12-infected 293 cells were separated on an 8% denaturing polyacrylamide gel. Cytoplasmic RNA prepared from uninfected 293 cells was used as a negative control (Lane 1). γ-32P-labeled DNA oligonucleotides complementary to 3' strand of mivaRNAI were used as probes. (B) Cleavage efficiency of mivaRNAs processed from Ad4, 5, 11 and 12 VA RNAI. Quantification was based on the signal intensity of Northern blotting.
Fig. 6. Detection of mivaRNAs produced from Ad4 and Ad5 VA RNAII. (A) Cytoplasmic RNAs prepared 24h post-infection from Ad4, 5-infected 293 cells were separated on an 8% denaturing polyacrylamide gel. Cytoplasmic RNA prepared from un-infected 293 cells was used as a negative control (Lane 1). γ-32P-labeled DNA oligonucleotides complementary to 3’ strand of mivaRNAII were used as probes. (B) Cleavage efficiency of mivaRNAs processed from Ad4 VA RNAII and Ad5 VA RNAII. Quantification was based on the signal intensity of Northern blotting.
2.3 mivaRNAI of Ad4, Ad5, Ad11 and Ad12 are all efficiently incorporated into RISC

As shown above, Ad4, Ad11 and Ad12 VA RNAI were efficiently cleaved into mivaRNAs. To determine whether these mivaRNAs are functionally associated with RISC complexes during infection, both cytoplasmic S15 extracts and nuclear extracts were prepared 24 hours post-infection from two 15-cm plates of 293-Ago2 cells infected at a multiplicity of 20 FFU/cell. Since the 293-Ago2 cell line has been engineered to overexpress a FLAG/HA-tagged Ago2 protein, Ago2-containing complexes (RISC) were immuno-purified from the extracts by anti-FLAG M2 agarose resin. RNAs were extracted from the immuno-purified complexes and detected by Northern blotting analysis (Fig. 7A and Fig. 8A). The presence of the 3’ and 5’ strands of the VA RNAI-derived mivaRNAs were detected using strand specific probes.

(A)

(B)

Fig. 7. Detection of RISC-associated mivaRNAs produced from 3’ strand of VARNAI. (A) Small RNAs in immuno-purified RISC from cytoplasmic extracts (Lane 5-6) and nuclear extracts (Lane 1-4) at 24h post-infection of Ad4, 5, 11 and 12 were detected by Northern blotting. Small RNAs extracted from un-infected 293-Ago2 cells was used as a negative control (Lane 5 for NE and Lane 10 for S15). DNA oligo nucleotides complementary to 3’ strand of mivaRNAI were used as probes. (B) RISC-associated 3’ strand of mivaRNAIs of Ad4, 5, 11 and 12 were quantified based on signal intensity.
As shown in Fig. 7A, we could see strong mivaRNA signals of all four serotypes in the RNA prepared from the S15 extracts, indicating that mivaRNAs produced by Ad4, Ad11 and Ad12 were efficiently incorporated into RISC complexes as Ad5. It appeared that Ad5 and Ad11 provided more RISC-associated mivaRNAs than Ad4 and Ad12 (Fig. 7B). The size of the Ad5 mivaRNAI was smaller than the other three, which agreed with our previous finding (Fig. 5A). A novel finding in our study was that we could also detect 3-26% of RISC-associated mivaRNAs in the nucleus (Fig. 7B).

As shown in Fig. 8A, mivaRNAs derived from the 5'-strand in Ad4 and Ad11 was also detected in RISC complexes prepared from S15 extracts, although in less amounts compared to Ad5. In addition, a certain amount of 5’ mivaRNAs existed in the nuclear extracts though the quantification was unreliable due to low quality of the experiment. Interestingly, Ad12 only produced 5’ RISC-associated mivaRNA that were detectable in the nucleus.

Fig.8. Detection of RISC-associated mivaRNAs produced from the 5’ strand of VARNAI. (A) Small RNAs in immuno-purified RISC from cytoplasmic extracts (Lane 6-9) and nuclear extracts (Lane 1-4) at 24h post-infection of Ad4, 5, 11 and 12 were detected by Northern blotting. Same RNA extract prepared from non-infected 293 cells was negative control (Lane 5 for NE and Lane 10 for S15). DNA oligonucleotides complementary to 5’ strand of mivaRNAI were used as probes. (B) RISC-associated 5’ strand mivaRNAIs of Ad4, 5, 11 and 12 were quantified based on signal intensity.
3 Discussion

Here we showed that four of human adenovirus serotypes; Ad4, Ad5, Ad11 and Ad12 (belonging to subgroup E, C, B and A (Table 1), respectively), all produced mivaRNAs, which were efficiently incorporated into RISC complexes. Thus, the suppression of the RNAi pathway by virus-associated RNA-derived small RNAs might be a common gene regulatory mechanism shared by different human adenovirus serotypes. Extensive DNA sequence analysis has shown that the VA RNA sequences vary considerably among serotypes. However, several highly conserved regions have been found to maintain the common stem-looped structure. Based on sequence alignments of 47 adenoviral serotypes, the six subgroups of viruses were arrange into three superfamilies: VA RNAI of subgroup A, C, F belong to superfamily 1 whereas subgroup B, E, D VA RNAI belong to superfamily 2, VAII of all serotypes are arranged into the last superfamily (Ma, et al.,1996). Both the 3’ and 5’ terminal stems of the VA RNAs from the different superfamilies display a high sequence similarity, indicating that adenoviral serotypes have the structural features to be substrates of Dicer and Ago. Furthermore, we have studied Ad5 VAI and Ad12 VA (superfamily 1), Ad4 VAI and Ad11 VA (superfamily 2) as well as Ad4 VAII and Ad5 VAII (superfamily 3), demonstrating that all the three superfamilies have the potential to generate small RNAs that may serve a function in the inhibition of RNAi. It has been shown that Ad5 VA RNAII is not essential for virus growth, at least not in tissue culture cells. However, the VA RNAII sequence is more conserved than that of VA RNAI, especially in the terminal stem region that generates the mivaRNAs (Ma, et al.,1996). This affinity could implicate an as yet unidentified and separate function of VA RNAII (Ma, et al.,1996). In this work we demonstrate that both Ad4 and Ad5 efficiently produce mivaRNAs from VA RNAII. Consistent with previous results the VAII mivaRNAs are preferentially incorporated into RISC (Xu, et al.,2007), indicating that the suppression of anti-viral RNAi might be a speculated stabilized function of VAII. The impact of the VA RNAs on either the RNAi machinery or the PKR pathway is probably controlled by the kinetics of accumulation and the concentration of the different forms of the VA RNAs and mivaRNAs. Quantitative data of VA RNAI expression in adenovirus-infected cells has only been published once in 1976 (Soderlund, et al.,1976). In that report the Ad2 VA RNAI was suggested to accumulate to approximately $10^8$ copies per infected HeLa cell. This figure appears to be unrealistically high in comparison of many other components expressed within cells. We therefore reinvestigated VA RNAI expression in Ad5 infected 293 cells under our experimental conditions. The results suggested that VA RNAI was expressed at a significantly lower level, around $2.6-4.0\times 10^6$ VA RNAI molecules per 293 cell. This number was 30-fold lower than the previously reported, but still should be counted as a high expression level owing to its great excess over cellular miRNA expression or any aberrantly expressed dsRNA, allowing VA RNAs to competitively bind to Dicer. Moreover, it should be noted that different serotypes express different amounts of VA RNA and mivaRNAs. Also, the efficiency of incorporation into RISC
varied between the serotypes. Noteworthy, Ad12 was found to produce much less VA RNAs compared to the other three tested serotypes.

The VA RNA derived mivaRNAs have generally been considered to interfere in the Dicer and RISC activities in the cytoplasmic (Andersson, et al.,2005; Xu, et al.,2007). In this study, approximately 3-26% of RISC-associated mivaRNAI was detected in the nucleus. Correspondingly, previous studies showed that the Ago2 proteins can be imported into nucleus from cytoplasm (Meister, et al.,2009). These indicate that the mivaRNAs might have a function connected with nuclear processes, like transcriptional control.

Adenovirus replication is divided into an early phase and a late phase. During the early phase nonstructural and regulatory proteins are primarily expressed, whereas the structural proteins required for viral assembly are primarily expressed at the late stage of infection. One lytic cycle takes around 30 hours and produces approximately $10^5 - 10^6$ new virions per cell. In this study, cytoplasmic RNA was extracted at 24h post-infection. However, we do not know whether the length of the replication vary among serotypes, suggesting a complication to the interpretation of the results. To improve the experiment cytoplasmic RNA should be prepared at various time-points to gain further insights to VA RNA expression by the different serotypes. Besides, detection of the adenoviral late proteins such as hexon is an approach to determine the late phase in a life cycle.

To detect small RNAs by Northern blotting, we used a relatively novel cross-linking method, which has been proved to enhance detection of small RNA molecules (<40nt) by up to 50-fold compared to conventional UV-cross-linking. This has been verified during our experimental work; for example, around 4-5.3% of Ad5 VA RNAII was cleaved by Dicer under our experimental conditions whereas 1-2% of Ad5 VA RNAII was detected to be cleaved using UV-crosslinking (Xu, et al.,2009). The comparison indicated that chemical-cross-linking is a better choice for studying siRNAs and miRNAs. The chemical cross-linking is mediated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which facilitates the formation of a covalent phosphoramidate bond between primary amine groups on the nylon membrane and 5’- terminal phosphates on the small RNAs (Pall, et al.,2007). However, since it is not known whether this method is dependent on a monophosphate at the 5’ end of RNA, the problem of bi-phosphates or tri-phosphates on the 5’ strand mivaRNA might cause problems in our quantifications. Thus, it remains for the future to establish the 5’ end phosphorylation status of the mivaRNAs, and the mechanistic details of the chemical cross-linking method.

Previous studies have suggested that the VA RNAI derived mivaRNAs may utilize the RNAi machinery to regulate gene expression of both the virus and the host (Fortes, et al.,2006). Here we have shown that Ad4, 5, 11 and 12 mivaRNAI are all efficiently incorporated into RISC and therefore may similarly as Ad5 mivaRNAI regulate gene expression. To gain further information about the structure of the mivaRNAs, the small RNA content in immuno-purified RISC should be sequenced from the various adenovirus infections. Further, the functional activity of the mivaRNA-associated RISC complexes should be established by various RISC assays. Also, a better characterization of Ad4 mivaRNAII may prove rewarding since it has
been speculated that only adenoviruses encoding two VA RNAs establish persistent infections (Allard, et al., 1992). The work also raises our interest in further research of the serotypes encoding single VA RNAs, such as Ad12, Ad40 and Ad41.

In summary, we demonstrated the high synthesis level of VA RNAI during a late adenovirus-infection in human cells. We showed that VA RNAI accumulated to approximately $2.6 \times 10^6$ molecules per Ad5-infected 293 cell under our experimental conditions. More importantly, four serotypes (Ad4, Ad5, Ad11 and Ad12), from different subgroups, were all shown to produce mivaRNAs that were efficiently incorporated into RISC, indicating that a shared VA RNA-mediated RNAi suppression mechanism may exist among the human adenoviruses.
4 Materials and Methods

4.1 Cell Culture

293 cells and 293-Ago2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100U/ml PEST at 37°C in 5% CO₂. All reagents used for cell culture were purchased from Gibco/BRL.

4.2 Virus Infection

Cells were infected with Ad4, Ad5, Ad11, and Ad12 at a multiplicity of 50 FFU/cell (for cytoplasmic RNA extraction) or 20 FFU/cell (for S15/NE extraction) in serum/PEST-free DMEM medium for 45 min. Then the infected cells were incubated in fresh DMEM/FCS 10% medium at 37°C in 5% CO₂ for 24 hours and harvested as described below. Cells used as un-infected control were treated the same way omitting the virus.

4.3 Cytoplasmic RNA Extraction

Infected cells from one 10-cm plate were treated with 600 μl of IsoB/NP40 (10 mM Tris-HCl [pH 7.9], 150 mM NaCl, 1.5 mM MgCl₂, 1% NP-40) on ice for 10 min, followed by phenol (600 μl) extraction twice, and Chisam (24:1 chloroform:isoamylalcohol, 600 μl) once. The RNA was finally precipitated by addition of isopropanol (600 μl) and 3M NaAc (pH 5.2, 30 μl) and stored in -20°C overnight. The concentration and purity of RNA were measured using a Nanodrop.

4.4 In vitro transcription of Ad5 VA RNAI

The standard VA RNAI for the quantification was prepared by in vitro transcription. The VA DNAI template was produced by PCR amplification (Phusion system, Finnzymes) on plasmid pHindB using a 5’ primer containing a T7 promoter. Transcription was mediated by T7 RNA polymerase (New England Biolabs) for 2 hours at 37°C. Pure VA RNAI was recovered by DNaseI treatment (twice) and purification of the small RNA from a polyacrylamide gel. The RNA concentration was measured by a Nanodrop and the copy number was calculated based on the known molecular weight and the Avogadro Constant.

4.5 Cytoplasmic S15 Extract and Nuclear Extract (NE) Preparation

293-Ago2 cells were cultured in two 15-cm plates for each Adenovirus serotype and infected as described above. Cells were collected and washed by phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄, pH 7.3). The pellets were resuspended in 6 volumes of hypotonic buffer A with dithiotreitol (DTT) (10 mM HEPES, pH7.9, 10 mM KCl, 1.5mM MgCl₂, 1 mM DTT) and allowed to swell on ice for 15 min. Cells were disrupted by passing the solution 30-40 times through a 23-gauge syringe needle. Nuclei were pelleted at 7,000×g and prepared for NE extraction. The supernatant supplemented with 0.1 volume
hypotonic buffer and 5% glycerol were cleared at 15,000×g for 60 min. Nuclei were resuspended in 1 volume of Buffer C (20mM HEPES KOH, pH7.9, 600mM KCl, 1.5mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1mM DTT) and disrupted by 10 strokes with 23-gauge syringe needle. NE was gained by swelling the solution on ice for 30 min and spinning at 13,000×g for 5 min. Both S15 extract and NE were quick-frozen in dry ice, and stored at -80°C. The protein concentration was quantified by the Bradford protein assay before immuno-purification.

4.6 Immuno-purification of RISC

Cytoplasmic S15 extract and NE from the different adenovirus infections were used for immuno-purification of RISC. Each sample (extract from two 15-cm plate of cells) was incubated with 30 μl of anti-FLAG M2 affinity resin beads (Sigma) on a roller shaker overnight at 4°C. The resin was washed with ice-cold NET-1 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2.5% Tween 20) three times and hypotonic buffer A (without DTT) once. The washed beads supplemented with one volume Buffer A and 50% glycerol were quick-frozen in dry ice and stored at -80°C. The immuno-purified RISC associated RNAs were extracted from the beads as described above followed by Northern blotting detection.

4.7 Fluorescence Forming Unit Assay

Wild type adenoviruses Ad4, Ad5, Ad11, and Ad12 were amplified in 293 cells. The viral titers were determined as fluorescent focus units (FFU) per ml. 6-cm plates of 293 cells were infected with serial dilutions of each serotype. Infectious viral particles were quantified 24 hours later by addition of primary anti-Ad2-hexon antibody from mouse (Millipore) and secondary FITC goat-anti-mouse IgG antibody (Millipore) and observation by fluorescence microscope.

4.8 Northern Blotting Analysis

Small RNAs were separated on a denaturing 8% polyacrylamide gel and semi-dry transferred to a Hybond NX membrane (Amersham) at 20V for 60 min. The RNAs were immobilized by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated chemical cross-linking (Pall, et al.,2007). The membrane was blocked using a hybridization buffer containing 6×SSC, 5×denhardts and 0.2% SDS before hybridization. Hybridization probes were γ-32P-labeled DNA oligonucleotides designed to match the 5’ or 3’ strand of the different mivaRNAs (Table 2). After hybridizing overnight at 42°C, the membrane was washed with 3× SSC-0.05% SDS solutions and exposed to a PhosphorImager. γ-32P-labeled pUC19 was used as size marker. RNA quantification was determined by radioactive signal intensity of volumes or band contours measured by Quantity One software.
Table 2. Oligonucleotide probes complementary to 3’ and 5’ strands of mivaRNAs

<table>
<thead>
<tr>
<th>Oligos complementary to 3’ strand of mivaRNAs</th>
<th>Oligos complementary to 5’ strand of mivaRNAs</th>
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<tbody>
<tr>
<td>3’ST Ad4VAI 5’-3’ AAAACGACCC GCCTCCGTAT CCTGGAGGTT TTG</td>
<td>5’ST Ad4VAI 5’-3’ GTTCGCTTAG CCTCCAGGCC ACGGAGTCGA GCC</td>
</tr>
<tr>
<td>3’ST Ad4VAlII 5’-3’ AAAAGAGGGG CTCGCTCCGT AACTGGAGAA GTC</td>
<td>5’ST Ad5VAI 5’-3’ CCTCGCAAT TTATCGACCA CGGAAGAGTG CCC</td>
</tr>
<tr>
<td>3’ST Ad5VAl 5’-3’ AAAAGGAGCG CTCCCCCGTT GTCTGACGTC GCA</td>
<td>5’ST Ad11VA 5’-3’ CGTTCACGTT CCTCCAGGCT ACGGAGTCGA GTC</td>
</tr>
<tr>
<td>3’ST Ad11VA 5’-3’ AAAACGACTC GATTCGTAT CCTGGATTTT TGT</td>
<td>5’ST Ad12VA 5’-3’ AAAAGACTCC TCTCCGTGTC TGGGTCTTT TGG</td>
</tr>
<tr>
<td>3’ST Ad12VA 5’-3’ AAAAGACTCC TCTCCGTGTC TGGGTCTTT TGG</td>
<td>5’ST Ad12VA 5’-3’ AAAAGACTCC TCTCCGTGTC TGGGTCTTT TGG</td>
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6 References


